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Yin-Chen-Hao-Tang ameliorates obstruction-induced hepatic apoptosis in rats

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Abstract

The accumulation of hydrophobic bile acids in the liver is considered to play a pivotal role in the induction of apoptosis of hepatocytes during cholestasis. Thus, factors that affect apoptosis may be used to modulate liver fibrosis. Yin-Chen-Hao-Tang (YCHT) decoctions have been recognised as a hepatoprotective agent for jaundice and various types of liver diseases. We used an experimental rat model of bile-duct ligation (BDL) to test whether YCHT plays a regulatory role in the pathogenesis of hepatic apoptosis. BDL-plus-YCHT groups received 250 or 500 mg kg^{-1} YCHT by gavage once daily for 27 days. YCHT significantly ameliorated the portal hypertensive state and serum TNF-α compared with the vehicle-treated control group. In BDL-plus-YCHT-treated rats, hepatic glutathione contents were significantly higher than than in BDL-only rats. BDL caused a prominent liver apoptosis that was supported by an increase in Bax and cytochrome *c* protein and increased expression of Bax and Bcl-2 messenger RNA. The normalising effect of YCHT on expression of Bax and Bcl-2 mRNA was dependent on the dose of YCHT, 500 mg kg^{-1} having the greater effect on both Bax and Bcl-2 of mRNA levels. Additionally, YCHT treatment down-regulated both hepatic caspase-3 and -8 activities of BDL rats. This study demonstrates the anti-apoptotic properties of YCHT and suggests a potential application of YCHT in the clinical management of hepatic disease resulting from biliary obstruction.

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Introduction

The role of apoptosis in normal liver biology and during liver diseases is well established (Patel & Gores 1995). Hepatocyte apoptosis and liver fibrosis are major features of a wide range of chronic liver injuries, including metabolic, viral, cholestatic and genetic disease. The failure of bile salt excretion in cholestasis leads to retention of hydrophobic bile salts within the hepatocytes (Greim et al 1972a, b) and causes apoptosis and/or necrosis (Myoshi et al 1999). Although the mechanism of bile-salt-mediated apoptosis is not completely understood, the involvement of antioxidants has been suggested by in-vitro studies, as well as by in-vivo studies using an animal model of bile-duct ligation (BDL) (Singh et al 1992).

Following a death-signal-induced apoptosis, mitochondrial cytochrome *c* is released into the cytoplasm (Gross et al 1999; Narita et al 1998), which activates caspase-9; this subsequently activates caspase-3 in concert with the cytosolic factor Apaf-1 (Li et al 1997). In addition, hepatocyte apoptosis during extrahepatic cholestasis is associated with an increase in Bax expression (Myoshi et al 1999).

Hepatic apoptosis occurs mostly in liver injury, whereas liver fibrosis is the response of hepatic stellate cells (HSCs) to this injurious process. It is attractive to speculate a possible link between hepatic apoptosis and liver fibrosis. Additionally, a number of studies have shown that administration of antioxidants or Chinese herbs leads to a decrease in hepatic transforming growth factor-β1 (TGF-β1) expression and severity of fibrosis in rats (Wasser et al 1998; Vendemiale et al 2001; Lee et al 2003, 2006). Therefore, modulation of hepatic apoptosis may inhibit liver fibrogenesis.

Plants contain abundant bioactive materials that interfere with cell-death pathways. Decoctions of the Chinese herbal medicine Yin-Chen-Hao-Tang (YCHT; the Japanese herbal medicine Inchin-ko-to (TJ-135)) have long been used in China and Japan as an anti-inflammatory, antipyretic, choleretic and diuretic agent for liver disorders and jaundice, and several studies provide clinical evidence for effectiveness in the treatment of various liver diseases. YCHT is

an aqueous extract from three herbs: *Artemisia capillaris* Thunb (Herba Artemisiae Capillaris, Yin-Cen-Hao), *Gardenia jasminoides* Ellis (Fructus Gardeniae, Zhi-zi) and *Rheum officinale* Baill (Radix Rhei Officinalis, Da-huang) with a ratio of 4:3:1 in weight. *A. capillaris* and *G. jasminoides* are effective for liver diseases, and *R. officinale* is a laxative (Komiyama et al 1976; Okuno et al 1988; Yamamoto et al 1996, 2000). Among components of *A. capillaris*, capillarisin can act as a choleretic (Komiyama et al 1976; Okuno et al 1988), and capillene and capillin inhibit apoptosis of hepatocytes induced by TGF-β (Yamamoto et al 1996). Genipin, an aglycone converted in the gut from geniposide in *G. fructus*, is proven to suppress Fas-induced liver injury in mice (Yamamoto et al 2000). Recently, YCHT was reported to suppress liver fibrosis induced in rats by choline-deficient diet (Sakaida et al 2003), but the mechanisms of such suppression are yet to be elucidated. Considering that YCHT seems to affect the signalling derived from TGF-β in hepatocytes (Yamamoto et al 1996), YCHT can regulate HSC activity. However the question may arise from this antifibrotic effect as to whether YCHT will ameliorate hepatic apoptosis after extrahepatic biliary obstruction. Accordingly, this study was designed to test the hypothesis that YCHT ameliorated hepatic apoptosis after BDL and thereby attenuated liver fibrosis.

Materials and Methods

Preparation of YCHT

YCHT extract powder consists of crude ingredients extracted from the following three medicinal herbs mixed in the ratio 4:3:1 by weight: *A. capillaris* Thunb (Herba Artemisiae Capillaris, Yin-Cen-Hao), *G. jasminoides* Ellis (Fructus Gardeniae, Zhi-zi) and *R. officinale* Baill (Radix Rhei Officinalis, Da-huang). YCHT was prepared by boiling the dried powder with distilled water for 5 h. The extract was filtered and freeze dried, and stored at 4°C. The extraction yield was approximately 10.1% (w/w). The dried extract was dissolved in distilled water before use. The HPLC chromatogram of YCHT and the three reference standards is shown in Figure 1. Three main index components of YCHT decoction were recognised by HPLC by comparing the retention times and UV spectra as 0.95% (w/w) geniposide, 0.14% capillarisin and 0.11% emodin. The analytical column was a Cosmosil C_{18} (250 mm × 4.6 mm internal diameter). The column temperature was maintained at 40°C. The HPLC mobile phase was a 65:35 mixture of water and acetonitrile containing phosphoric acid (0.03% v/v), at a flow rate of 1.0 mL min^{-1}; 20 μL of solution was injected into the HPLC system for analysis. Peaks correlating with geniposide, capillarisin and emodin represent *G. jasminoides* Ellis, *A. capillaris* Thunb, and *R. officinale* Baill, respectively.

Biliary obstruction and animal treatment

Adult male Sprague–Dawley rats weighing between 220 and 250 g were used in all experiments. Hepatic damage with apoptosis was produced by ligation of the common bile duct, as described previously (Yang et al 2001). In brief, under ether anaesthesia, the common bile duct was ligated with 3-0 silk and sectioned between the ligatures. The midline abdominal incision was closed with catgut. Sham-operated rats had their bile duct exposed but not ligated or sectioned. All rats were caged at 24°C with a 12:12 h light–dark cycle and were allowed free access to food and water. Animal studies were approved by the Animal Experiment Committee of Chang-Gung University and were conducted humanely. Four groups of rats (6–8 rats in each group) were used in this experimental

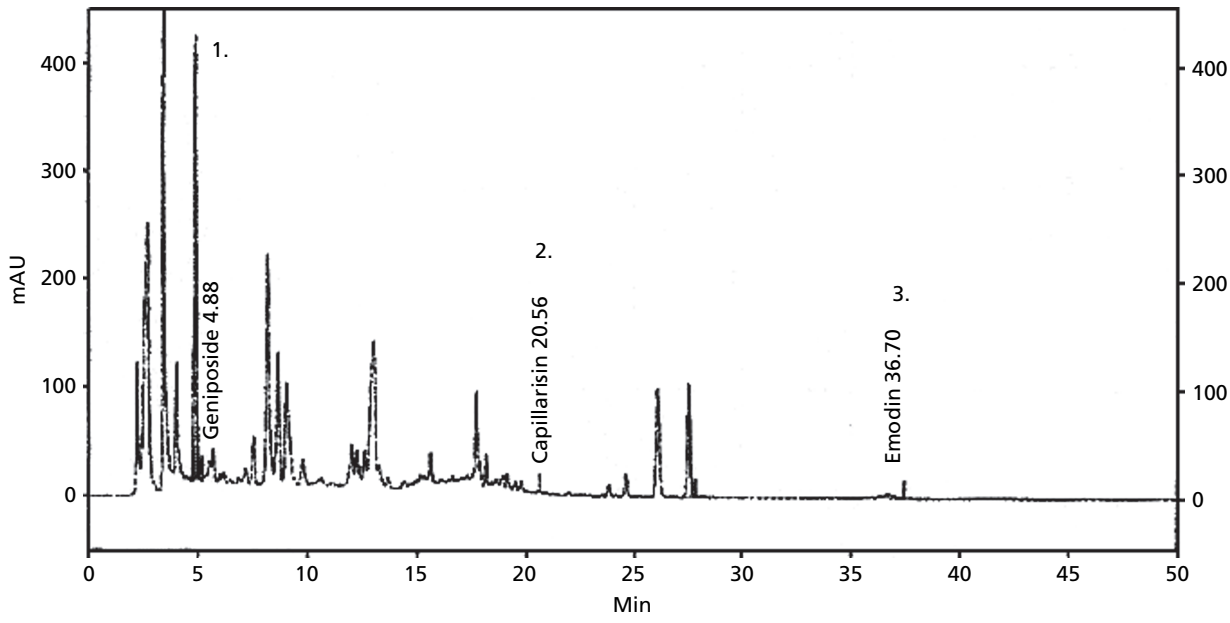

Figure 1 HPLC-UV chromatogram of raw YCHT, with the three reference standards. The three main index components of YCHT were identified by comparing the retention times and UV spectra with standards of the compound. 1 = geniposide; 2 = capillarisin; 3 = emodin.

protocol. Two groups of rats were given YCHT, 250 or 500 mg kg^{-1} daily orally by gavage, for 27 days. Control (i.e. sham-operated) rats were given equivalent volumes of saline; BDL-only rats were given equivalent volumes of drug vehicle (distilled water).

Haemodynamic studies

Haemodynamic studies were performed 28 days after BDL. Under ketamine anaesthesia (100 mg kg^{-1} i.m.), a tracheostomy was performed to keep the airway patent. A femoral artery catheter was inserted to monitor the arterial pressure and heart rate, and blood samples were obtained from the femoral artery at the end of the study. The abdomen was then opened by a midline incision, and the portal vein was cannulated via a small ileal vein for measurement of portal pressure. The tip of the catheter was placed in the distal part of the superior mesenteric vein. The rectal temperature was maintained at 37°C by use of a heating lamp. All pressures were measured and recorded with a multi-channel recorder (model TA 240, Gould, Cupertino, CA, USA).

Measurement of hepatic glutathione

Hepatic levels of glutathione (GSH) were measured using a glutathione-400 colorimetric assay kit (Calbiochem Co., San Diego, CA, USA) and a spectrophotometer, as per our previous study (Lee et al 2006). Liver tissue was homogenised with 2 mL 10% (w/v) metaphosphoric acid solution at 4°C. Samples were then centrifuged at 3000 *g* for 10 min at 4°C. A 50 μl aliquot of the centrifuged supernatant was added to assay buffer provided by the manufacturer to give a total volume of 200 μl. The reaction mixtures were incubated at 25°C for 30 min, and the absorbance at 412 nm was measured by a spectrophotometer. The values of unknown samples were determined from a standard curve plotted by assaying known concentrations of GSH. The amounts of GSH were expressed as nmol (mg protein)$^{-1}$.

Histopathology assay and plasma tumour necrosis factor-α measurement

The liver tissue was fixed in 10% formalin, embedded in paraffin, cut into 5 μm thick sections, stained with Masson's trichrome, and examined under the light microscope by an experienced pathologist. Blood was obtained for serum biochemical analysis. The concentrations of TNF-α in the serum were measured using a commercial ELISA kit (R & D Systems Inc., MN, USA).

Caspase-3 and -8 activities

Caspase activities were determined using a colorimetric assay kit (BioSource International, Inc., Camarillo, CA, USA). Fresh liver tissues were homogenised with a Teflon homogeniser in lysis buffer containing 25 mmol L^{-1} Hepes (pH 7.4), 5 mmol L^{-1} EDTA and 2 mmol L^{-1} dithiothreitol. The lysates were clarified by centrifugation, and supernatants were used for enzyme assays. Caspase activities were measured by colorimetry using specific substrates (Asp-Glu-Val-Asp-pNA for caspase-3, and Ile-Glu-Thr-Asp-pNA for caspase-8) according to the manufacturer's protocol. The reaction mixtures were incubated at 37°C for 90 min, and the absorbance measured by a spectrophotometer at 405 nm. Capsase activity was expressed as % relative to the control group.

Western blot analysis of Bax, Bcl-2 and cytochrome *c*

Freshly isolated liver tissue was homogenised in buffer containing 10 mmol L^{-1} Tris-HCl (pH 7.4), 1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mmol L^{-1} PMSF and 5 mmol L^{-1} EDTA. Cytosolic extracts were obtained as the supernatant after centrifugation at 12500 *g* for 45 min at 4°C in a preparative ultracentrifuge (XL-90; Beckman, Palo Alto, CA, USA). The protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard. Aliquots of protein (60 or 120 μg per lane) were loaded onto 10% polyacrylamide gels and electrophoresed. Proteins were transferred to nitrocellulose membranes. The membrane was blocked overnight with buffer and then incubated with primary antibodies for 1 h using 1:1000 dilutions of mouse monoclonal anti-cytochrome *c* (15 kDa) (Pharmingen, San Diego, CA, USA), mouse polyclonal anti-Bax (23 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Bcl-2 (28 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or goat polyclonal anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed three times in Tris-buffer solution for 15 min, and incubated with 1:5000 dilution of alkaline-phosphatase-conjugated goat anti-mouse IgG (Calbiochem, San Diego, CA, USA) as the second antibody for 1 h. The protein was visualised with an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL, USA). The membranes were finally exposed to X-ray film for 1 min. The relative expression of various proteins was quantified by densitometric scanning using an image analysis system.

Reverse transcription polymerase chain reaction (RT-PCR) amplification of Bax and Bcl-2

Approximately 300 mg liver was snap-frozen in liquid nitrogen and homogenised in 3 mL Triazol solution (BRL, Gaithersburg, MD, USA). Total cellular RNA was extracted according to the manufacturer's instructions. A 1 μg aliquot of total RNA from each sample was resuspended in 20 μl reaction buffer. Once the reaction mixture reached 42°C 400 U of reverse transcriptase was added to each tube, and the samples were incubated for 30 min at 42°C. Reverse transcription was stopped by denaturing the enzyme at 99°C. The reaction mixture was diluted with distilled water to a final volume of 50 μl. Commercially available PCR primers for Bax, Bcl-2 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Purigo Biotech, Inc. (Taipei, Taiwan, R.O.C.) and contained the following sequences: Bax (369 bp), sense 5′-TCCACCAAGAAGCTGAGCGAGT-3′, antisense 5′-CACAAAGATGGTCACTGTCTGCC-3′; Bcl-2 (472 bp), sense 5′-GCTACGAGTGGGATACTGG-3′, antisense 5′-GTGTGCAGATGCCGGTTCA-3′ and G3PDH (209 bp), sense 5′-CCCTTCATTGACCTCAACTACATGG-3′, antisense 5′-CATGGTGGTGAAGACGCCAG-3′. The

primer pairs used for the amplification of Bax (Oshiro et al 2002), Bcl-2 (Valks et al 2003) and G3PDH (Lee et al 2003) have been described previously. The amplified PCR products were electrophoresed at 75 V through 2% agarose gel for 1 h. The PCR products were size fractionated on agarose gels and visualised by ethidium-bromide staining. The relative amount of messenger RNA (mRNA) transcripts was photographed using Pharmacia Biotech System (D&R, Israel) and scanned by the Imaging Master. Densitometric analysis of the captured image was normalised for G3PDH.

Statistics

The results are expressed as means ± s.e.m. Statistical analysis was performed using one-way analysis of variance followed by Student–Newman–Keuls' multiple-range test. $P < 0.05$ was considered significant.

Results

Systemic and splanchnic haemodynamics

Twenty-eight days after BDL, rats receiving vehicle had significantly higher portal pressure than sham-operated rats. After 27 days' YCHT administration, lower portal pressure were noted in BDL rats treated with 250 or 500 mg kg^{-1} YCHT daily (Table 1).

YCHT improved plasma TNF-α levels and hepatic GSH levels of BDL rats

Twenty-seven days after BDL, plasma TNF-α (Table 1) levels were significantly higher than in control rats. In BDL rats receiving YCHT (250 mg kg^{-1}), TNF-α levels were lower than in rats with BDL alone. The concentration of GSH in the control group was about two times than that in rats with BDL alone. Rats receiving concomitant YCHT administration showed less decrease in liver GSH levels. These results show that YCHT may have antioxidant properties, although GSH levels were reduced lower than in the control group (Table 1).

YCHT improved liver histopathology of BDL rats

Histological examination showed a significant change in the profile of collagen fibre deposition in the liver sections of rats after treatment with YCHT compared with rats that received BDL alone (Figure 2). Liver sections from the control rats showed no fibrosis (Figure 2A). After BDL surgery, livers showed typical histological changes characterised by central±central architecture disruption and bridge fibrosis formation (Figure 2B). The BDL rats that were treated with YCHT (250 mg kg^{-1}) (Figure 2C) showed significantly less histological collagen accumulation compared with the BDL-only group.

Effect of YCHT treatment on hepatic caspase-3 and -8 activities

Figure 3 shows the pattern of hepatic apoptosis caused by BDL, as determined by the more than two-fold increase in caspase-3 activity and three-fold increase in caspase-8 activity above control-group values 27 days after rats received BDL. In contrast, liver samples from rats treated with YCHT showed substantially attenuated caspase-3 (Figure 3A) and caspase-8 (Figure 3B), as indicated by reduction of the enzyme activities.

Table 1 Effects of bile-duct ligation (BDL) and YCHT on haemodynamic profiles, plasma TNF-α and hepatic glutathione levels

Group	Control	BDL only	BDL + YCHT 250 mg kg^{-1}	BDL + YCHT 500 mg kg^{-1}
n	6	8	8	8
Mean arterial pressure (mmHg)	125 ± 3	103 ± 4*	105 ± 4*	105 ± 5*
Heart rate (beats min^{-1})	387 ± 13	399 ± 15	384 ± 11	391 ± 15
Portal pressure (mmHg)	7.3 ± 0.3	18.5 ± 0.7*	16.1 ± 0.4*,#	15.4 ± 0.5*,#
TNF-α (pg mL^{-1})	1.4 ± 0.4	22.0 ± 1.2*	11.5 ± 1.5*,#	9.7 ± 1.0*,#
Glutathione (μmol (g liver)$^{-1}$)	5.0 ± 0.5	2.4 ± 0.6*	3.5 ± 0.5*,#	3.3 ± 0.4*,#

Values are mean ± s.e.m. (one-way analysis of variance followed by Student–Newman–Keuls' test). *$P < 0.05$ compared with control group; #$P < 0.05$ compared with BDL group.

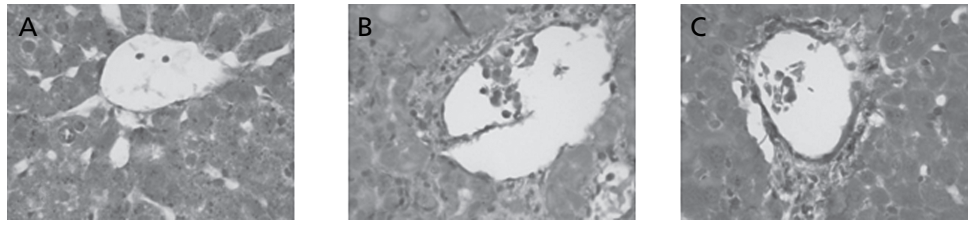

Figure 2 Light micrographs of rat liver sections of control rats (A) and after BDL treatment alone (B) and with concomitant YCHT administration (250 mg kg^{-1} per day) (C) for 27 days. Paraffin-embedded sections were stained with Masson's trichrome. Original magnification ×200.

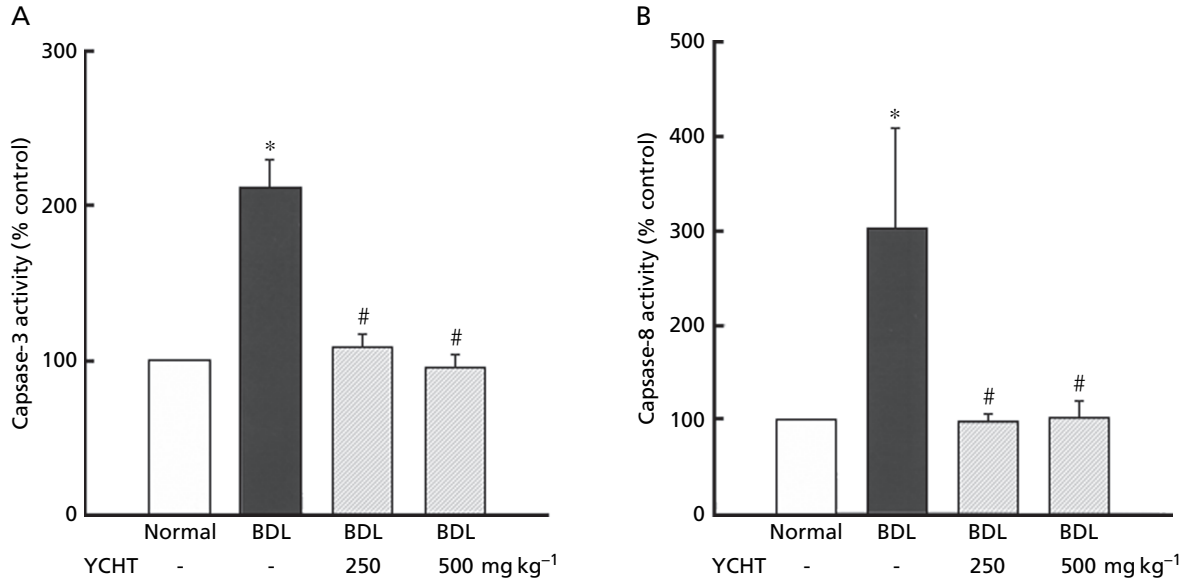

Figure 3 Effect of YCHT on hepatic caspase-3 (A) and caspase-8 (B) activities in control rat, in rats after BDL alone, and after BDL plus YCHT (250 or 500 mg kg^{-1} per day) for 27 days. *$P < 0.05$ vs control rats; $^{\#}P < 0.05$ vs rats receiving BDL alone.

Effect of YCHT treatment on hepatic Bax, Bcl-2 and cytochrome *c* protein content after BDL surgery

The expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 (Figure 4) was measured in BDL rats and YCHT-treated BDL rats. Following BDL, total Bax protein initially increased. Following BDL for 27 days, Bax and Bcl-2 proteins were increased in liver homogenates. Treatment of rats with YCHT decreased the expression of Bax in BDL rats. In parallel to the results of Western blot analysis, the intensity of Bax increased in BDL-only rats but decreased dramatically after treatment with YCHT. Anti-apoptotic Bcl-2 protein is known to be expressed in BDL-induced hepatic apoptosis as an adaptive mechanism to resist hepatocyte damage induced by bile salts. The Bcl-2 protein contents of liver homogenates were significantly decreased in rats receiving BDL plus YCHT (500 mg kg^{-1}) compared with those receiving BDL alone.

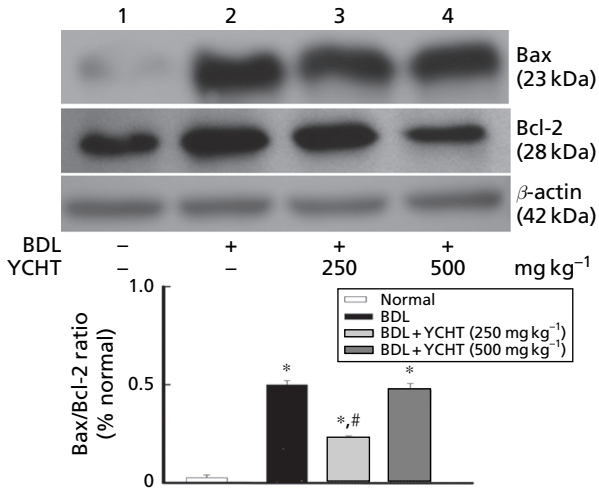

Figure 4 Western blot analysis of hepatic Bax and Bcl-2 protein contents of control rats, in rats after BDL alone, and with BDL plus YCHT (250 or 500 mg kg^{-1}) for 27 days. Liver homogenate fractions (60 μg protein per lane) were analysed for immunoreactivity with antibodies recognising Bax or Bcl-2. The membranes were also probed with an antibody recognising β-actin to ensure equal protein loading in the respective lanes. The gels are representative of three experiments from three separate animals. *$P < 0.05$ vs control rats; $^{\#}P < 0.05$ vs rats receiving BDL alone.

Cytochrome *c* is localised in the space between the inner and outer mitochondrial membrane. Considering that BDL induces apoptotis, it would be expected to trigger the release of cytochrome *c* from membrane into cytosol. The representative Western blots (Figure 5) show an increase in cytochrome *c* levels in cytosolic fractions from rats receiving BDL alone. Rats receiving BDL plus YCHT exhibited an accordant decrease in cytochrome *c* levels. Equal loading of protein was confirmed by the blot with antibody against β-actin.

YCHT reduced levels of Bax and Bcl-2 transcript in BDL rats' livers

Bax and Bcl-2 mRNA transcripts isolated from the livers of control rats, rats receiving BDL alone or BDL plus YCHT were examined by RT-PCR to determine the relative changes in gene expression. Livers from control rats showed basal levels of Bax and Bcl-2 expression, while increased levels were observed with 27 days' BDL. Concomitant treatment with YCHT caused a marked dose-dependent reduction in the level of expression of the Bax (Figure 6A) and Bcl-2 transcript (Figure 6B).

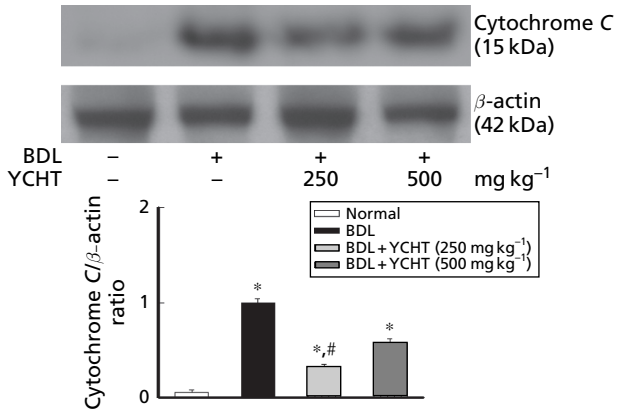

Figure 5 Western blot analysis of hepatic cytochrome *c* protein contents of control rats, in rats after BDL alone, and after BDL plus YCHT (250 or 500 mg kg^{-1} per day) for 27 days. Liver cytosol fractions (120 μg protein per lane) were analysed for immunoreactivity with an antibody recognising cytochrome *c*. The membranes were also probed with an antibody recognising β-actin, to ensure equal protein loading in the respective lanes. The gels are representative of three experiments from three separate animals. $^{*}P < 0.05$ vs control rats; $^{\#}P < 0.05$ vs rats receiving BDL alone.

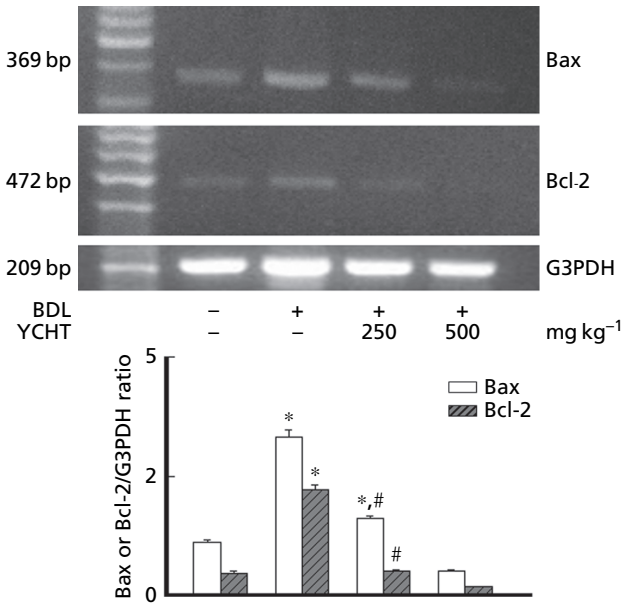

Figure 6 Expression of hepatic Bax and Bcl-2 mRNA relative to G3PDH in whole liver of control rats, after BDL alone, and after BDL plus YCHT (250 or 500 mg kg^{-1} per day) for 27 days. Hepatic samples were obtained at the same time points. Using cDNA equivalents of 10 ng RNA, samples were amplified for 28 cycles using specific Bax and Bcl-2 primers. Bax and Bcl-2 mRNA expression is normalised against G3PDH mRNA expression. Values are expressed as mean ± s.e.m. for three separate experiments. $^{*}P < 0.05$ vs control rats; $^{\#}P < 0.05$ vs rats receiving BDL alone.

Discussion

The aim of this study was to determine the possible molecular effects of YCHT on hepatic injury induced by bile salts in BDL rats. YCHT is a Chinese herbal medicine reported to have anti-fibrotic (Sakaida et al 2003; Inao et al 2004) and anti-apoptotic (Yamamoto et al 1996) activities. Apoptotic signalling within cells is mainly via two pathways: the death receptor pathway (i.e. the extrinsic pathway) and the mitochondrial pathway (i.e. the intrinsic pathway) (Guicciardi & Gores 2005).

It has been demonstrated that the BDL rat model shows almost no generation of toxic intermediates, no massive hepatocyte necrosis and no significant inflammation (Muriel et al 1994). BDL induces a type of liver damage that is aetiologically and pathogenetically different from the experimental liver damage induced by poisons such as carbon tetrachloride (Kountouras et al 1984). The BDL model therefore allows the detection of anti-apoptotic drug effects. Using the BDL model or toxic bile salts in primary hepatocyte culture, several researchers have examined the anti-fibrotic and hepatoprotective effects of apoptosis-modulating agents. Hydrophilic bile acids such as ursodeoxycholic acid and tauroursodeoxycholic acid reduce the incidence of apoptosis in hepatocytes induced by toxic bile salts (Benz et al 1998, 2000); antioxidants exert the same effect (Yerushalmi et al 2001). The results of our current study demonstrate that the treatment of BDL rats with YCHT prevents hepatocellular apoptosis in-vivo. Although the hepatoprotective and antifibrotic effects of YCHT or its constituents have been demonstrated in various cell types and animal models (Yamamoto et al 1996, 2000; Sakaida et al 2003), the molecular mechanisms by which YCHT protects the hepatocyte from toxic-bile-salt-induced apoptosis are described for the first time in this study. According to previous studies, YCHT has been reported to suppress liver injury in mice induced by Fas (Yamamoto et al 2000) and hepatic fibrosis in rats induced by a choline-deficient L-amino-acid-defined diet (Sakaida et al 2003), thioacetamide (Imanishi et al 2004) or repeated injections of carbon tetrachloride or pig serum (Inao et al 2004). Moreover, the beneficial effect of YCHT on postoperative biliary atresia has been shown; improvement in markers of hepatic fibrosis such as hyaluronic acid, prolyl hydroxylase, procollagen III peptide and type VI collagen has been reported (Kobayashi et al 2001).

Regarding the mechanism(s) by which YCHT suppresses liver injury, it has been shown that YCHT prevents apoptosis of cultured rat hepatocytes (Yamamoto et al 1996, 2000) and suppresses proliferation and fibrogenesis of cultured rat HSCs (Imanishi et al 2004; Inao et al 2004) and those of human HSC lines (Sakaida et al 2003).

BDL rats also display increased plasma levels of bile acids with depletion of endogenous antioxidant (Singh et al 1992). It is widely accepted that the severity of hepatic damage in individuals or in experimental animals with biliary obstruction is causally associated with the extent of intrahepatic oxidative stress (Baron & Muriel 1999; Ljubuncic et al 2000). Increased levels or accelerated generation of reactive oxygen species and toxic degradative products of lipid peroxidation induce hepatic cell death. Based on these findings, we have postulated that the antioxidative properties of YCHT may contribute to the protection of the liver from damage induced by toxic bile salts in BDL rats. The current results obtained with the long-term treatment of BDL rats with YCHT for

27 days show that YCHT significantly affects the levels of GSH, a marker of hepatic antioxidant defence activation during the fibrogenic process. Moreover, YCHT administration resulted in a reduction of TNF-α levels and replacement of GSH stores, suggesting an effective role of this agent against oxidative stress.

We have confirmed the possible relationship between fibrosis and apoptosis. Elevation of the Bax protein level occurs in many clinically relevant settings where cell death occurs. Bcl-2 is an apoptosis-suppressing factor that heterodimerises with Bax, neutralizing its protective effects. When Bcl-2 is present in excess, it may induce possible compensatory mechanism(s) that protect hepatocytes from apoptosis. In contrast, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed cell death. The total amount of rat liver bile acids is increased after BDL. The expression of Bax and Bcl-2 mRNA and protein levels significantly decreased, thereby reducing the Bax/Bcl-2 ratio after 250 mg kg^{-1} per day YCHT treatment of BDL rats. Moreover, the expression of Bcl-2 protein was not consistent with YCHT doses. Under treatment with the higher dose of YCHT, Bcl-2 protein significantly decreased in hepatic tissue. In addition, we hypothesise that the possible antioxidant effects of YCHT lead to a reduction in oxidative stress following BDL administration, with a subsequent decrease in Bax activation. Thereafter, the Bcl-2 protein content was also reduced because of a lesser degree of hepatic apoptosis. In cases of extrahepatic cholestasis, the use of YCHT may provide an effective prophylactic and therapeutic tool by inhibiting apoptosis in hepatocytes. The elucidation of the properties of the factors involved in the interactions of bile salts in hepatocytes may uncover the mechanism of action of the anti-apoptotic effects of YCHT.

Conclusions

YCHT is a choleretic and jaundice agent that has been used in traditional Chinese medicine for centuries. Our results indicate that treatment with YCHT after the establishment of BDL-induced hepatic apoptosis in rats significantly ameliorates the apoptosis. However, the decoction may confer only partial protection when there is ongoing toxic injury to the liver.

References

Baron, V., Muriel, P. (1999) Role of glutathione, lipid peroxidation and antioxidants on acute bile-duct obstruction in the rat. *Biochim. Biophys. Acta* **1472**: 173–80

Benz, C., Angermuller, S., Tox, U., Kloters-Plachky, P., Riedel, H. D., Sauer, P., Stemmel, W. (1998) Effect of tauroursodeoxycholic acid on bile-acid-induced apoptosis and cytolysis in rat hepatocytes. *J. Hepatol*. **28**: 99–106

Benz, C., Angermuller, S., Otto, G., Sauer, P., Stremmel, W., Stiehl, A. (2000) Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. *Eur. J. Clin. Invest*. **30**: 203–209

Greim, H., Trülzsch, D., Roboz, J., Dressler, K., Czygan, P., Hutterer, F., Schaffner, F., Popper, H. (1972a) Mechanism of cholestasis. 5. Bile acids in normal rat livers and in those after bile duct ligation. *Gastroenterology* **63**: 837–845

Greim, H., Trülzsch, D., Czygan, P., Rudick, J., Hutterer, F., Schaffner, F., Popper, H. (1972b) Mechanism of cholestasis. 6. Bile acids in human livers with or without biliary obstruction. *Gastroenterology* **63**: 846–850

Gross, A., McDonnell, J. M., Korsmeyer, S. (1999) Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev*. **13**: 1899–1911

Guicciardi, M.E., Gores, G. J. (2005) Apoptosis: a mechanism of acute and chronic liver injury. *Gut* **54**: 1024–1033

Imanishi, Y., Maeda, N., Otogawa, K., Seki, S., Matsui, H., Kawada, N., Arakawa, T. (2004) Herb medicine Inchin-ko-to (TJ-135) regulates PDGFB-dependent signaling pathways of hepatic stellate cells in primary culture and attenuates development of liver fibrosis induced by thioacetamide administration in rats. *J. Hepatol*. **41**: 242–250

Inao, M., Mochida, S., Matsui, A., Eguchi, Y., Yulutuz, Y., Wang, Y., Naiki, K., Kakinuma, T., Fujimori, K., Nagoshi, S., Fujiwara, K. (2004) Japanese herbal medicine Inchin-ko-to as a therapeutic drug for liver fibrosis. *J. Hepatol*. **41**: 584–591

Kobayashi, H., Horikoshi, K., Yamataka, A., Lane, G. J., Yamamoto, M., Miyano, T. (2001) Beneficial effect of a traditional herbal medicine (inchinko-to) in postoperative biliary atresia patients. *Ped. Surg. Inter*. **17**: 386–389

Komiyama, T., Tsukui, M., Oshio, H. (1976) Studies of 'Inchinko'. I. Capillarisin, a new choleretic substance. *Yakugaku Zasshi* **96**: 841–854

Kountouras, J., Billing, B. H., Scherer, P. J. (1984) Prolonged bile duct obstruction: A new experimental model for cirrhosis in the rat. *Br. J. Exp. Pathol*. **65**: 305–311

Lee, T. Y., Wang, G. J., Chiu, J. H., Lin, H. C. (2003) Long-term administration of *Salvia miltiorrhiza* ameliorates the carbon tetrachloride-induced chronic hepatic fibrosis in rats. *J. Pharm. Pharmol*. **55**: 1561–1568

Lee, T. Y., Chang, H. H., Wang, G. J., Chiu, J. H., Yang, Y. Y., Lin, H. C. (2006) Water-soluble extract of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats. *J. Pharm. Pharmol*. **58**: 659–665

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., Wang, X. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**: 479–489

Ljubuncic, P., Tanne, Z., Bomzon, A. (2000) Evidence of a systemic phenomenon for oxidative stress in cholestatic liver disease. *Gut* **47**: 710–716

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurements with folin phenol reagent. *J. Biol. Chem*. **193**: 265–275

Muriel, P., Suarez, O. R., Gonzalez, P., Zuniga, L. (1994) Protective effect of *S*-adenosyl-L-methionine on liver damage induced by biliary obstruction in rats: a histological, ultrastructural and biochemical approach. *J. Hepatol*. **21**: 95–102

Myoshi, H., Rust, C., Roberts, P. J., Burgart, L. J., Gores, G. J. (1999) Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. *Gastroenterology* **117**: 669–677

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., Tsujimoto, Y. (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. *Proc. Natl. Acad. Sci. USA* **95**: 14681–14686

Okuno, I., Uchida, K., Nakamura, M., Sakurai, K. (1988) Studies on choleretic constitutions in *Artemisia capillaris* THUNB. *Chem. Pharmacol. Bull*. **36**: 769–775

Oshiro, T., Shiraishi, M., Muto, Y. (2002) Adenovirus mediated gene transfer of antiapoptotic protein in hepatic ischemia-reperfusion

injury: the paradoxical effect of Bcl-2 expression in the reperfused liver. *J. Surg. Res*. **103**: 30–36

Patel, T., Gores, G. J. (1995) Apoptosis and hepatobiliary disease. *Hepatology* **21**: 1725–1741

Sakaida, I., Tsuchiya, M., Kawaguchi, K., Kimura, T., Terai, S., Okita, K. (2003) Herbal medicine Inchin-ko-to (TJ-135) prevents liver fibrosis and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. *J. Hepatol.* **38**: 762–769

Singh, S., Shackleton, G., Ah-Sing, E., Chakraborty, J., Bailey, M. E. (1992) Antioxidant defenses in the bile duct ligated rat. *Gastroenterology* **103**: 1625–1629

Valks, D. M., Kemp, T. J., Clerk, A. (2003) Regulation of Bcl-xL expression by H_2O_2 in cardiac myocytes. *J. Biol. Chem.* **278**: 25542–22547

Vendemiale, G., Grattagliano, I., Caruso, M. L., Serviddio, G., Valentini, A. M., Pirrelli, M., Altomare, E. (2001) Increased oxidative stress in dimethylnitrosamine-induced liver fibrosis in the rat: effect of N-acetylcysteine and interferon-alpha. *Toxicol. Appl. Pharmacol*. **175**: 130–139

Wasser, S., Ho, J. M., Ang, H. K., Tan, C. E. (1998) Salvia miltiorrhiza reduces experimentally-induced hepatic fibrosis in rats. *J. Hepatol*. **29**: 760–771

Yamamoto, M., Ogawa, K., Morita, M., Fukuda, K., Komatsu, Y. (1996) The herbal medicine Inchin-ko-to inhibits liver cell apoptosis induced by transforming growth factor β1. *Hepatology* **23**: 552–559

Yamamoto, M., Miura, N., Ohtake, N., Amagaya, S., Ishige, A., Sasaki, H. (2000) Genipin, a metabolite derived from the herbal medicine Inchin-ko-to, and suppression of Fas-induced lethal liver apoptosis in mice. *Gastroenterology* **118**: 380–389

Yang, Y. Y., Lin, H. C., Huang, Y. T., Lee, T. Y., Lee, W. C., Hou, M. C., Lee, F. Y., Chang, F. Y., Lee, S. D. (2001) Adaptive vasodilatory response after octreotide treatment. *Am. J. Physiol*. **281**: G117–123

Yerushalmi, B., Dahl, R., Devereaux, M. W., Gumpricht, E., Sokol, R. J. (2001) Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. *Hepatology* **33**: 616–626